Available online at www.sciencedirect.com

ScienceDirect

Virology 370 (2008) 122–129

VIROLOGY

www.elsevier.com/locate/yviro

Removal of a N-linked glycosylation site of classical swine fever virus strain Brescia E^{rns} glycoprotein affects virulence in swine

I. Fernandez Sainz [a], L.G. Holinka [a], Z. Lu [c], G.R. Risatti [b], M.V. Borca [a,*]

[a] *Plum Island Animal Disease Center, ARS, USDA/ARS/NAA, P.O. Box 848, Greenport, NY 11944-0848, USA*
[b] *Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269, USA*
[c] *Plum Island Animal Disease Center, DHS, Greenport, NY 11944, USA*

Received 19 June 2007; returned to author for revision 8 August 2007; accepted 21 August 2007
Available online 29 September 2007

Abstract

E^{rns} glycoprotein, along with E_1 and E_2, is one of the three envelope glycoproteins of classical swine fever virus (CSFV). E^{rns} is a heavily glycosylated protein involved in several functions, including virus attachment and entry to target cells, production of neutralizing antibodies, and virulence. The role of added glycans to CSFV strain Brescia E^{rns} on virus virulence was assessed in swine. A panel of virus mutants was constructed and used to investigate whether the removal of each of seven putative glycosylation sites in the E^{rns} glycoprotein would affect viral virulence in swine. Only N269A/Q substitution rendered attenuated viruses (N1v/N1Qv) that, unlike BICv and other mutants, produced a transient infection in swine characterized by mild symptoms and decreased virus shedding. Notably, N1v efficiently protected swine from challenge with virulent BICv at 3 and 21 days post-infection suggesting that glycosylation of E^{rns} could be modified for development of CSF live-attenuated vaccines.
Published by Elsevier Inc.

Keywords: Virulence; Pathogenesis; Attenuation; Protection; Glycoproteins; Glycosylation; Classical swine fever virus

Introduction

Classical swine fever (CSF) is a highly contagious disease of swine caused by CSFV, an enveloped, positive sense, single-stranded RNA Pestivirus in the *Flaviviridae* family (Becher et al., 2003). The 12.5 kb CSFV genome contains a single open reading frame that encodes a 3898-amino acid polyprotein, that after co- and post-translational processing by cellular and viral proteases yields 11 to 12 cleavage products (NH_2-Npro-C-E^{rns}-E_1-E_2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (Rice, 1996).

Structural components of the virion include the capsid (C) protein and glycoproteins E^{rns}, E_1, and E_2. E_1 and E_2 are anchored to the envelope at their carboxyl termini and E^{rns} loosely associates with the viral envelope (Thiel et al., 1991) Modifications introduced into these glycoproteins appear to have an important effect on CSFV virulence (Meyers et al., 1999; Risatti et al., 2005a,b, 2006, 2007a,b; Van Gennip et al., 2004). Even though glycosylation of E^{rns}, E_1, or E_2 proteins may play a significant role in the lifecycle of CSFV, the function of added oligosaccharides is not well known. We have recently reported that glycosylation of E_2 plays a role in virulence of CSFV strain Brescia (Risatti et al., 2007b). A single mutation, N805A, in one of the six N-linked glycosylation sites in E_2 rendered a completely attenuated virus.

E^{rns} from Pestiviruses possess several biological activities. E^{rns} lacks a typical membrane anchor and is secreted from infected cells but is associated with mature virions (Rumenapf et al., 1993), it contains ribonuclease activity (Schneider et al., 1993), its C-terminal domain controls translocation across eukaryotic cell membranes (Langedijk, 2002), and has a role in the inhibition of double-stranded RNA-induced cell responses (Iqbal et al., 2004). An important feature is that E^{rns} is heavily

* Corresponding author. Fax: +1 631 323 3006.
E-mail addresses: Ignacio.Fernandez-Sainz@ars.usda.gov (I. Fernandez Sainz), lauren.holinka@ars.usda.gov (L.G. Holinka), zlu@piadc.ars.usda.gov (Z. Lu), guillermo.risatti@uconn.edu (G.R. Risatti), manuel.borca@ars.usda.gov (M.V. Borca).

0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2007.08.028

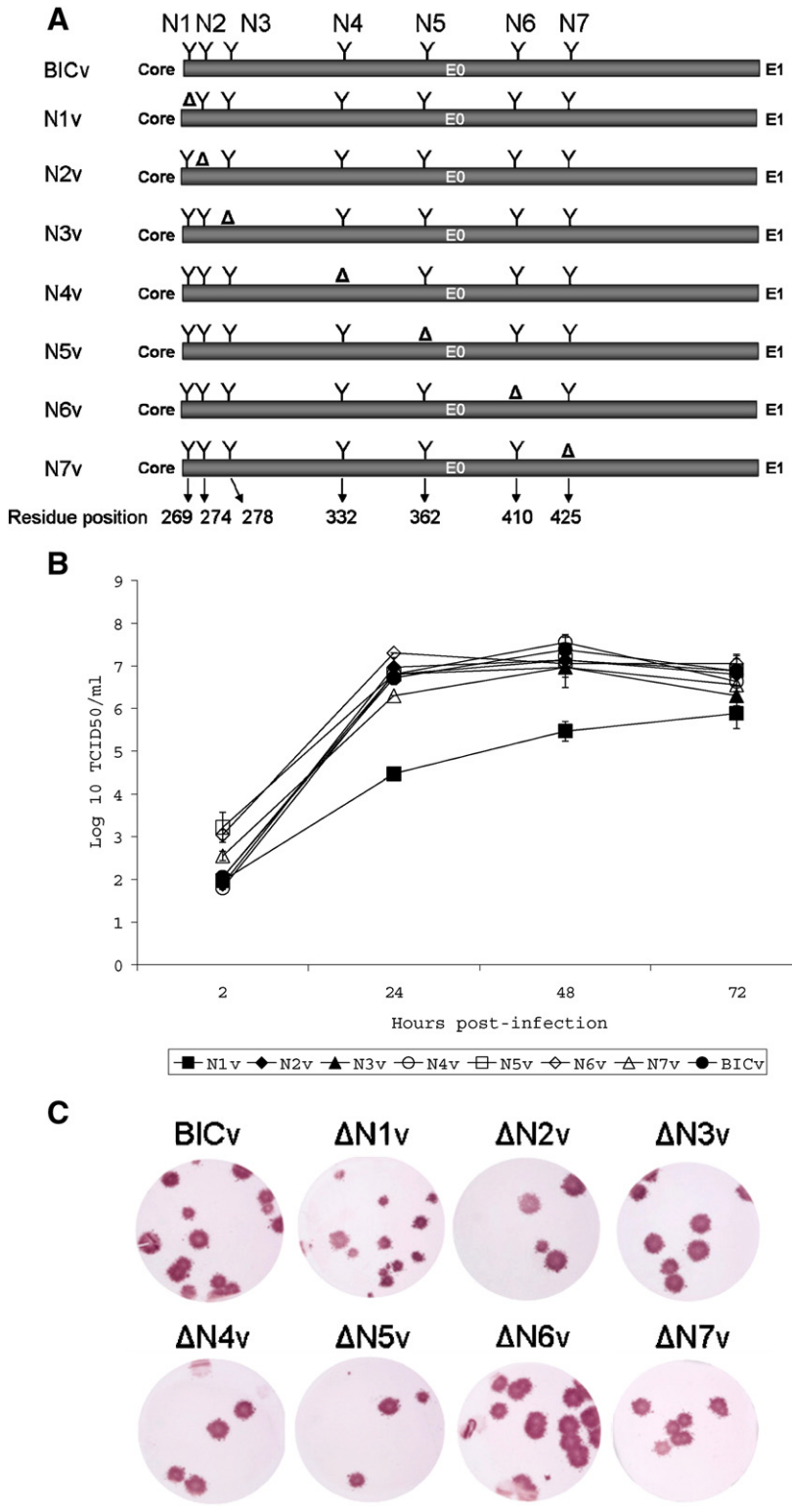

Fig. 1. (A) Schematic representation of glycosylation mutants of Classical Swine Fever Virus E^{rns} protein, generated by site-directed mutagenesis of a cDNA full-length clone pBIC. Wild-type E^{rns} glycoprotein is shown at the top. Y: putative glycosylation sites. Mutants were named with an N (N-linked glycosylation) followed by a number that represents the relative position of putative glycosylation sites within E^{rns} amino acid sequence (N1, N2, N3, N4, N5, N6 and N7) starting from the amino terminus. (B) *In vitro* growth characteristics of E^{rns} individual glycosylation mutants and parental BICv. Primary swine macrophage cell cultures were infected (MOI = 0.01) with each of the mutants or BICv and virus yield titrated at times post-infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: $\geq \log_{10}$ 1.8 TCID50/ml. (C) Plaque formation of E^{rns} glycosylation mutants and BICv. SK6 monolayers were infected, overlaid with 0.5% agarose and incubated at 37 °C for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and stained by immunohistochemistry with mAb WH303 (Edwards et al., 1991).

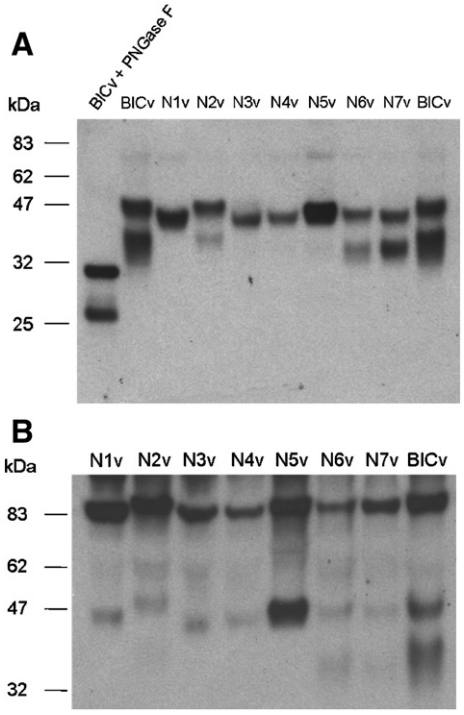

Fig. 2. E^{rns} glycoprotein analysis was done by Western immunoblot of reducing (A) or non-reducing (B) 12% SDS-PAGEs. SK6 monolayers were infected (MOI=1) with each of the mutants or parental BICv and harvested 48 h post-infection. CSFV E^{rns} was detected with mAb 16/15 (kindly provided by Prof. J-H Thiel, Institut für Virologie, Frankfurter, Germany).

glycosylated (Moormann et al., 1990) with N-linked glycans counting for up to half of the apparent molecular weight (Branza-Nichita et al., 2004). In this study, we used oligonucleotide site-directed mutagenesis of the highly virulent CSFV strain Brescia E^{rns} gene to construct a panel of seven individual glycosylation mutants (at positions N269, N274, N278, N332, N362, N410 and N425 of the CSFV polyprotein). These mutants were applied to investigate whether the removal of each of these glycosylation sites in the E^{rns} glycoprotein could affect viral infectivity and virulence in swine. We found that one of the seven single mutations introduced in E^{rns}, N269 substituted by A or Q, renders an attenuated virus (N1v/N1Qv) with decreased virus replication and shedding in infected swine.

Results

Replication of glycosylation mutants in vitro

Infectious RNA was *in vitro* transcribed from full-length ICs of the CSFV Brescia strain or a set of glycosylation mutants and used to transfect SK6 cells. Mutants referred to as N1, N2, N3, N4, N5, N6, and N7 represent each of seven putative glycosylation sites starting from the N terminus of E^{rns} (Fig. 1A). Nucleotide sequences of the rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted glycosylation sites were reflected in rescued viruses.

In vitro growth characteristics of mutant viruses N1v, N2v, N3v, N4v, N5v, N6v, and N7v were evaluated relative to parental BICv in a multistep growth curve performed in swine macrophage cell cultures (Fig. 1B). All single glycosylation site mutants, except N1v, exhibited growth characteristics indistinguishable from BICv. N1v exhibited a lower rate of growth and a 5- to 10-fold decrease in the final virus yield (Fig. 1B). Additionally, N1v exhibited a reduction in plaque size relative to BICv and other mutants in SK6 cell cultures (Fig. 1C).

Analysis of relative electrophoretic mobility of E^{rns} in virus mutants

E^{rns} glycosylation status of BICv and mutant viruses was analyzed in lysates of SK6-infected cells by Western immunoblots using monoclonal antibody (mAb) 16/15. Assuming that differences in E^{rns} mobility among mutants and parental viruses are likely due to the number of carbohydrate moieties attached to the protein, we observed that E^{rns} from single mutants N1v, N3v, N4v, N6v and N7v migrated further than E^{rns} from mutants N2v, N5v or parental BICv (Fig. 2A). The data suggested that N1, N3, N4, N6 and N7 sites, but not N2 and N5 sites in BICv E^{rns}, are targeted for the addition of glycans in swine SK6 cells. Furthermore, the lack of glycosylation at N1, N3, N4, N6 and N7 sites does not appear to alter the formation of E^{rns} homodimers as revealed by Western blot analysis of SK6 cell lysates run on non-reducing SDS–PAGEs (Fig. 2B). Bands with apparent molecular masses of 48 and 80 kDa,

Table 1
Swine survival and fever response following infection with CSFV E^{rns} glycosylation mutants and parental BICv

Virus	No. of survivors/ total no.	Mean time to death (days±SD)	Fever		
			No. of days to onset (days±SD)	Duration no. of days (days±SD)	Maximum daily temperature °F (±SD)
BICv	0/4	8.5 (1)	4.5 (0.5)	3.5 (0.5)	106.4 (0.9)
N1v	5/5b[a]	–	5.6 (0.5)[b]	3.2 (0.5)	104.6 (1.1)
N2v	0/2	6.5 (0.7)	5 (0.4)	3 (0)	105.2 (0.3)
N3v	0/2	7.5 (2.1)	4 (0)	4 (1.4)	105.8 (0.9)
N4v	0/2	9 (2.8)	4 (0)	5.5 (2.1)	106.4 (0.5)
N5v	0/2	7.5 (0.7)	4 (0)	3.5 (0.7)	106.4 (0.6)
N6v	0/2	8.5 (2.1)	4 (0)	4.5 (2.1)	106.5 (0.1)
N7v	0/2	11 (0)	4 (0)	7 (0)	106.9 (0.9)
N1Qv	3/3	–	6 (0)	2 (0)	105.1 (1.3)

[a] The original experiment performed with 2 animals were repeated with 3 more individuals. Presented results represent data from both experiments.
[b] Only 2 out of 5 animals showed fever.

corresponding to E^{rns} monomers and homodimers, respectively, as described elsewhere (Weiland et al., 1999; Branza-Nichita et al., 2004), were observed in all infected lysates (Fig. 2B). An expected shift in electrophoretic mobility of these bands was observed in lysates from SK6 cells infected with N1v, N3v, N4v, N6, and N7v. BICv-infected SK6 cell extracts treated with (PNGase F) showed a 27- to 29-kDa band, the expected unglycosylated molecular mass of E^{rns} (Fig. 2A) as described before (Branza-Nichita et al., 2004).

Mutant N1v lacks determinants necessary for CSFV virulence in swine

Virulence of E^{rns} glycosylation mutants was analyzed in swine. An initial screening was established in 16 pigs (10- to 12-week-old, 40-lb commercial breed) that were randomly allocated into 8 groups of 2 animals each, and were intranasally (IN) inoculated with 10^5 $TCID_{50}$ of one of each single glycosylation mutant, or virulent BICv. After inoculation, swine were monitored daily for clinical disease and changes in body temperature (Risatti et al., 2005a). Blood, nasal swabs and tonsil scrapings were collected at times post-infection, processed, titrated, and analyzed as described by Risatti et al. (2007b).

While BICv exhibited a characteristic virulent phenotype with animals dying by days 8–9 post-infection, animals inoculated with N1v survived the infection presenting a short period of mild fever and transient diarrhea. Conversely, animals infected with N2v, N3v, N4v, N5v, N6v and N7v showed CSF with clinical presentation and severity similar to that observed in animals inoculated with BICv (Table 1). Mutations were

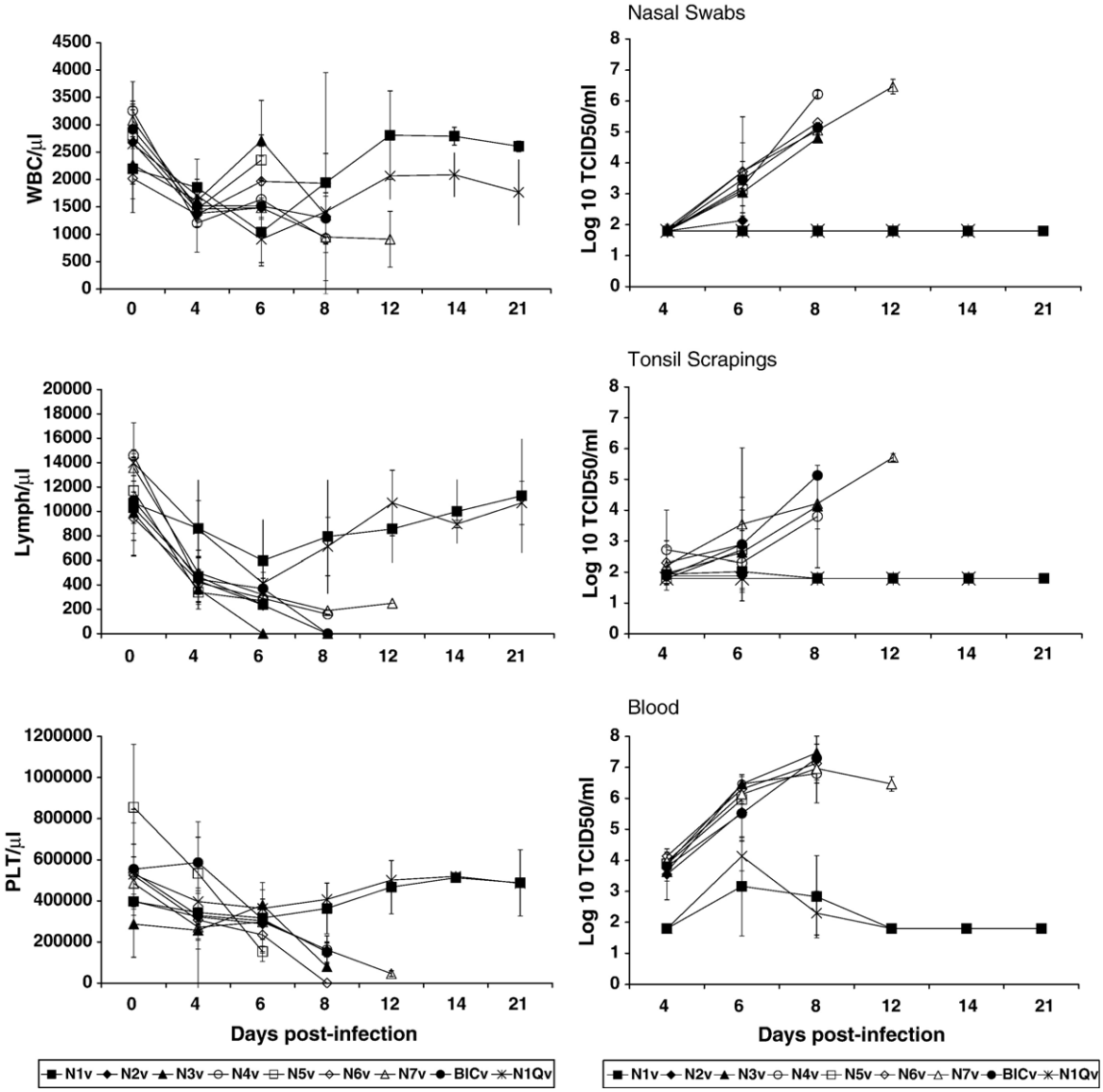

Fig. 3. Hematological data (panels on the left) and virus titers (panels on the right) of blood samples from animals infected with CSFV E^{rns} glycosylation mutants and parental BICv. Peripheral white blood cell (WBC), lymphocytes (Lymph) and platelet (PLT) counts are expressed as counts/μl of blood. Data represent means and standard deviations from at least two animals per inoculated virus. Virus titers in nasal swabs, tonsil scrapings and blood are expressed as the mean $\log_{10}$ TCID50/ml and standard deviations from at least two animals per inoculated virus. Sensitivity of virus detection: $\geq \log_{10}$ 1.8 TCID50/ml.

confirmed by sequencing of glycosylation sites in viruses isolated from infected animals.

Total white blood cell, lymphocyte and platelet counts dropped by 4 to 6 days post-infection (DPI) in animals inoculated with N2v, N3v, N4v, N5v, N6v, N7v and BICv and kept declining until death. In opposition, a transient decrease of cell counts was observed in animals inoculated with N1v (Fig. 3).

Furthermore, N1v was attenuated and showed a restricted *in vivo* replication capability. Viremia in N1v inoculated animals was transient and significantly reduced by 10^3 to 10^4 from that observed in pigs infected with BICv and other glycosylation mutants (Fig. 3). A similar pattern was observed for virus titers from tonsil samples, and with no virus titers detected in nasal samples obtained from N1v-infected animals (with a sensitivity of $\geq \log_{10}$ 1.8 $TCID_{50}$/ml) (Fig. 3). Interestingly, all the other mutants retained the same capability of causing severe disease in swine as parental BICv, showing that *in vivo* E^{rns} functions are retained and not influenced by the lack of glycans at positions N274, N278, N332, N362, N410 and N425.

To determine if N1v attenuation is, beside the lack of glycosylation in residue N269, due to an intrinsic characteristic of the N to A substitution, a N269Q virus (N1Qv), harboring a more conserved residue substitution was constructed. N1Qv showed *in vitro* growth characteristics similar to N1v (data not shown). Animals inoculated via the intranasal route with 10^5 $TCID_{50}$ of N1Qv developed, as the N1v-infected animals did, a transient disease characterized by mild fever, depression and diarrhea. Hematological and virological values of these animals also resembled those of the N1v-infected animals (Fig. 3).

N1v mutant protects pigs against lethal CSFV challenge

Since N1v showed a restricted *in vivo* growth as observed with live-attenuated CSFV vaccine CSICv (Risatti et al., 2005b), the ability of N1v to induce protection against virulent BICv was assessed in early and late vaccination–exposure experiments. Three groups of pigs (n=4) received intranasal inoculation of 10^3 $TCID_{50}$ N1v or were mock-inoculated. At 3 DPI or 21 DPI, animals were challenged with BICv along with mock-vaccinated animals. Mock-vaccinated animals developed CSF and died or were euthanized in extremis by 12 days post-challenge (DPC) (Table 2). Conversely, N1v induced complete protection by 3 and 21 DPI. All pigs survived infection and remained clinically normal through the observation period. Mock-inoculated pigs showed high virus titers in the blood and oronasal cavity starting 4 DPC (Table 2). Virus was detected in blood and tonsils in one animal challenged at 3 DPI, by 4 DPC; while no virus was detected in samples obtained from animals challenged at 21 DPI. Additionally, no significant hematological changes were recorded in any of the challenged animals (data not shown).

Discussion

CSFV strain Brescia E^{rns} glycoprotein contains 7 putative N-linked glycosylation sites (http://www.cbs.dtu.dk/services/) (Moormann et al., 1990) (Fig. 1A). Comparative sequence analysis of E^{rns} glycoproteins showed that these sites (N269, N274, N278, N332, N362, N410 and N425 of CSFV polyprotein) are highly conserved among CSFVs. Five of them, at positions N269, N278, N332, N362, and N410 are also highly conserved among Pestiviruses (BVDV type I and II, and BDV) (data not shown), implying a significant role for these sites in the viral cycle of all *Pestiviruses*. In this study, we used oligonucleotide site-directed mutagenesis of the highly virulent CSFV strain Brescia E^{rns} gene to construct a panel of glycosylation mutants. These mutants were investigated to determine whether the removal of each of these glycosylation sites in the E^{rns} glycoprotein could affect viral infectivity and virulence in swine. Results demonstrated that one of these sites (N269) has a major role in virulence and protection and, interestingly, not all of the sites seem to be essential for *in vitro* or *in vivo* infectivity.

Electrophoretic mobility analysis of the E^{rns} glycoprotein shows that amino acid residues N269 (N1v), N278 (N3v), N332 (N4v), N410 (N6v) and N425 (N7v) are used for carbohydrate addition (Fig. 2). *In vitro* growth characteristics and virus progeny yields of these mutants assessed in primary swine macrophage cultures, a CSFV natural target cell, were comparable

Table 2
Detection of virus in nasal swabs, tonsil scrapings, and blood samples obtained from N1v vaccinated animals after challenge of with virulent BICv at 3 and 21 DPI

Challenge group	Sample	Days post-challenge						
		C[a]	4	6	8	12	14	21
3 DPI	Nasal	0/4[b]	0/4	0/4	0/4	0/4	0/4	0/4
	Tonsil	0/4	1/4 (1.97)[c]	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	1/4 (1.97)	0/4	0/4	0/4	0/4	0/4
21 DPI	Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Mock-vaccinated	Nasal	0/3	0/3	3/3 (4.1)	3/3 (6.4)	3/3 (5.2)[d]		
	Tonsil	0/3	0/3	3/3 (4.2)	3/3 (5.4)	3/3 (4.7)		
	Blood	0/3	3/3 (2.6)	3/3 (6.6)	3/3 (7.5)	3/3 (7.4)		

[a] C, day of challenge.
[b] Number of animals positive for virus isolation over total number of challenged animals.
[c] Numbers in parentheses indicate average virus titers expressed as $\log_{10}$ $TCID_{50}$/ml for 4 animals.
[d] All mock-vaccinated animals died or were euthanized in extremis by 12 DPC.

to that of parental BICv, except for mutant N1v (N269A) that demonstrated delayed growth kinetics (Fig. 1B). Suggestive of a role for CSFV E^{rns} glycosylation patterns in virus attachment, entry, and/or exit from infected cells was the small plaque phenotype exhibited by single mutant N1v in which a substantial plaque size reduction relative to parental BICv was observed in infected SK-6 cells (Fig. 1C). Similarly, loss of one specific *N*-linked glycosylation site (G4) from the hemagglutinin–neuraminidase protein (HN) of Newcastle Disease Virus (NDV) yields plaques in cultured cells of considerably smaller size (Panda et al., 2004), while three other single mutants (G1, G2, and G3) produced plaques comparable to the size of the parental virus. Interestingly, G4 virus was considerably attenuated in chickens (Panda et al., 2004; McGinnes and Morrison, 1995). In the case of CSFV, we have observed that other BICv-derived viruses containing recombinant E2 protein (Risatti et al., 2005a, 2007a,b) have shown reduced plaque size as well. Like N1v described here, those recombinant viruses were also attenuated in swine. Cleavage and glycosylation patterns of the hemagglutinin and neuraminidase genes of H5 avian influenza viruses have also been shown to affect pathogenicity in chickens (Deshpande et al., 1987; Horimoto and Kawaoka, 1994; Hulse et al., 2004). As described for NDV, the mechanisms by which these patterns affect virus virulence are unknown.

N1v within E^{rns} rendered attenuated viruses with restricted *in vivo* replication ability (Table 1 and Fig. 3). Unlike the acute fatal disease induced by BICv, infections caused by N1v mutants were sub-clinical in swine and characterized by decreased viral replication and reduced virus shedding. Interestingly, mutants N2v, N3v, N4v, N5v, N6v and N7v retained the same capability of causing severe disease in swine as parental BICv, showing that *in vivo* E^{rns} functions are retained and not influenced by the lack of glycans at positions, N274, N278, N332, N362, N410 and N260, suggesting a specific function for glycan residues added at position N269. The mutation in N1v seems to be stable, since the N269A substitution was observed in viruses isolated after four passages in SK6 cells as well as in viruses isolated from infected pigs at 6 DPI, where the virus is expected to undergo an unknown number of replication/passage events (data not shown).

E^{rns} has previously been reported to play a role in CSFV virulence. Mutations at residues H297 and H346 abrogate E^{rns} RNase activity, resulting in attenuation of parental CSFV strain Alfort/Tübingen (Meyers et al., 1999). Although we have not evaluated the RNase activity of N1v/N1Qv E^{rns}, their attenuating mutation, N269A, is not involved in the E^{rns} RNase motif. Importantly, it has been shown that RNase activity in *Pestiviruses* is not affected by N-glycosylation inhibitors (Branza-Nichita et al., 2004). Therefore, if CSFV attenuation caused by mutations at residues H297 and H346 is mediated by loss in RNase activity, attenuation in N1v/N1Qv should be mediated by different mechanisms.

There is no information on the role of glycosylation in *Pestivirus* virulence. We have recently reported that amino acid substitution N805A within the CSFV polyprotein E2 gene led to complete attenuation of the virulent strain Brescia (Risatti et al., 2007a). This result, along with the data presented here, demonstrates a significant role of glycosylation in CSFV virulence *in vivo*. In BVDV it has been observed that inhibitors of N-glycosylation do not affect E^{rns} native conformation as judged by retention of RNase activity of the protein, or dimerization; however secretion of E^{rns} into the media is severely impaired, suggesting that trafficking of the protein within the secretory pathway is affected (Branza-Nichita et al., 2004). Impairment of viral secretion, mediated by α-glucosidase inhibitors, results in reduction of infectivity of newly BVDV released viral particles (Durantel et al., 2001). With the exception of N1v, we have observed that removal of individual N-glycosylation sites in the CSFV E^{rns} protein do not affect virus replication *in vitro* or *in vivo*. These E^{rns} mutants retained the same phenotypic features of parental BICv when tested in primary swine cultured cells or in swine.

In summary, we determined the utilization of five out of seven putative N-glycosylation sites in the CSFV strain Brescia E^{rns} protein. Individual N-linked glycosylation sites are not essential for *in vitro* or *in vivo* virus infectivity, and one site, N269, was shown to be involved in attenuation of BICv. The effective protective immunity elicited by N1v suggests that glycosylation of E^{rns} could be modified for the development of live-attenuated vaccines.

Materials and methods

Viruses and cells

Swine kidney cells (SK6), free of BVDV, were cultured in Dulbecco's minimal essential media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO) (Risatti et al., 2005a). CSFV Brescia strain was propagated in SK6 cells and used for the construction of an infectious cDNA clone (Risatti et al., 2005a). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described by Zsak et al. (1996). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Viral infectivity was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibody WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was ≥ 1.8 $TCID_{50}$/ml. Plaque assays were performed using SK6 cells in 6-well plates (Costar). SK6 monolayers were infected, washed, overlaid with 0.5% agarose and incubated at 37 °C for 3 days. Plates were fixed with 50% (vol./vol.) ethanol–acetone and stained by immunohistochemistry with mAb WH303 (Risatti et al., 2005a).

Construction of CSFV E^{rns} glycosylation mutants

Each of the E^{rns} N-linked glycosylation sites, predicted by analysis tools from the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/), were mutated in a full-length

infectious clone (IC) of the virulent Brescia isolate (BICv) (Risatti et al., 2005a). N to A (N to Q for N1Qv) substitutions were introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX), performed per manufacturer's instructions and using the following primers (only 5′→3′ forward primer sequences are shown; italics represent base substitutions, mutated codons are underlined); N1v: TACCAACCTGTTGCAGCCGAA *GC*TATAACTCAATGGAACCTGAGT; N1Qv: TACCAACCTGTTGCAGCCGAA*CA G*ATAACTCAATGGAACCTGAGT; N2v: GCCGAAAATATAACTCAATGG*GCC*C T GAGTGACAAGCGTACCAAT; N3v: ACTCAATGGAACCTGAGTGAC*GCC*GGTA CCAATGGTATCCAGCAC; N4v: ATGGATGCCAGCGAGGGGACA *GCC*TATACGT GCTGTAAGTTACAG; N5v: GACCCCTGGATACAGTTGATG*GC*TAGAACCCAAG CAAACTTGGCA; N6v: ACTGGCTGCAAGAAAGGGAAA*GC*TTTTTCTTTTGCGG GTACAGTT; N7v: ATAGAGGGCCCATGTAATTTC*GCC*GTTTCTGTTGAGGATA TCTTA. Mutants were referred to as N1, N2, N3, N4, N5, N6 and N7, representing each of the seven putative glycosylation sites starting from the N terminus of E^{rns} (N269, N274, N278, N332, N362, N410 and N425 of the CSFV polyprotein, respectively) (Fig. 1A).

In vitro rescue of CSFV Brescia and glycosylation mutants

Full-length genomic clones were linearized with *Srf*I and *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). RNA was precipitated with LiCl and transfected into SK6 cells by electroporation at 500 V, 720 Ω, 100 W with a BTX 630 electroporator (BTX, San Diego, CA) as described by Risatti et al. (2005a). Cells were seeded in 6-well plates and incubated for 4 days at 37 °C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at −70 °C.

Full-length clones and *in vitro* rescued viruses were completely sequenced with CSFV specific primers by the dideoxynucleotide chain-termination method (Sanger et al., 1977). Viruses recovered from infected animals were sequenced in the mutated area. Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a PRISM 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled with the Phrap software program (http://www.phrap.org). The final DNA consensus sequence represented an average 5-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Western blot analysis

Glycosylation status of the E^{rns} glycoprotein of BICv and mutant viruses was analyzed in lysates of SK6-infected cells by Western immunoblots. CSFV E^{rns} was detected using monoclonal antibody (mAb) 16/15 (kindly provided by Prof. J.-H. Thiel, Institut für Virologie, Justus Liebig Universität Giessen, Frankfurter, Germany). Cell extract preparation, Western immunoblots, and Peptide N-glycosidase F (PNGase F) digestions were performed as described by Risatti et al. (2007a).

Animal infections

Each of the E^{rns} glycosylation mutants was screened for its virulence phenotype in swine relative to virulent Brescia virus. Swine used in all animal studies were 10- to 12-week-old, 40-lb commercial breed pigs inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type virus. For screening, 16 pigs were randomly allocated into 8 groups of 2 animals each, and pigs in each group were inoculated with one of the E^{rns} glycosylation mutants or BICv. Clinical signs (anorexia, depression, fever, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the experiment as previously described (Risatti et al., 2007a).

For protection studies, 12 pigs were randomly allocated into 3 groups of 4 animals each. Pigs in groups 1 and 2 were inoculated with N1v, and pigs in group 3 were mock-infected. At 3 DPI (group 1) or 28 DPI (group 2), animals were challenged with BICv along with animals in group 3. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood, serum, nasal swabs and tonsil scrapings were collected at times post-challenge, with blood obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer) for total and differential white blood cell counts. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, CA).

Acknowledgments

We thank the Plum Island Animal Disease Center Animal Care Unit staff for excellent technical assistance. We thank Ms. Melanie Prarat for editing the manuscript.

References

Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., Konig, M., Schweizer, M., Stalder, H., Schirrmeier, H., Thiel, H.J., 2003. Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. Virology 311, 96–104.

Branza-Nichita, N., Lazar, C., Dwek, R.A., Zitzmann, N., 2004. Role of N-glycan trimming in the folding and secretion of the pestivirus protein E(rns). Biochem. Biophys. Res. Commun. 319, 655–662.

Deshpande, K.L., Fried, V.A., Ando, M., Webster, R.G., 1987. Glycosylation affects cleavage of an *H5N2* influenza virus hemagglutinin and regulates virulence. Proc. Natl. Acad. Sci. U. S. A. 84, 36–40.

Durantel, D., Branza-Nichita, N., Carrouee-Durantel, S., Butters, T.D., Dwek, R.A., Zitzmann, N., 2001. Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. J. Virol. 75, 8987–8998.

Edwards, S., Moennig, V., Wensvoort, G., 1991. The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. Vet. Microbiol. 29, 101–108.

Horimoto, T., Kawaoka, Y., 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol. 68, 3120–3128.

Hulse, D.J., Webster, R.G., Russell, R.J., Perez, D.R., 2004. Molecular determinants within the surface proteins involved in the pathogenicity of *H5N1* influenza viruses in chickens. J. Virol. 78, 9954–9964.

Iqbal, M., Poole, E., Goodbourn, S., McCauley, J.W., 2004. Role for bovine viral diarrhea virus E^{rns} glycoprotein in the control of activation of beta interferon by double-stranded RNA. J. Virol. 78, 136–145.

Langedijk, J.P., 2002. Translocation activity of C-terminal domain of pestivirus E^{rns} and ribotoxin L3 loop. J. Biol. Chem. 277, 5308–5314.

McGinnes, L.W., Morrison, T.G., 1995. The role of individual oligosaccharide chains in the activities of the HN glycoprotein of Newcastle disease virus. Virology 212, 398–410.

Meyers, G., Saalmuller, A., Buttner, M., 1999. Mutations abrogating the RNase activity in glycoprotein E(rns) of the pestivirus classical swine fever virus lead to virus attenuation. J. Virol. 73, 10224–10235.

Moormann, R.J., Warmerdam, P.A., van der Meer, B., Hulst, M.M., 1990. Nucleotide sequence of hog cholera virus RNA: properties of the polyprotein encoded by the open reading frame spanning the viral genomic RNA. Vet. Microbiol. 23, 185–191.

Panda, A., Elankumaran, S., Krishnamurthy, S., Huang, Z., Samal, S.K., 2004. Loss of N-linked glycosylation from the hemagglutinin–neuraminidase protein alters virulence of Newcastle disease virus. J. Virol. 78, 4965–4975.

Reed, L.J., Muench, H.A., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

Rice, C.M., 1996. *Flaviviridae*: the viruses and their replication, In: Knipe, B.N.F.D.M., Howley, P. (Eds.), Fundamental Virology, Third ed. Lippincott Raven, Philadelphia, pp. 931–959.

Risatti, G.R., Borca, M.V., Kutish, G.F., Lu, Z., Holinka, L.G., French, R.A., Tulman, E.R., Rock, D.L., 2005a. The E_2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J. Virol. 79, 3787–3796.

Risatti, G.R., Holinka, L.G., Lu, Z., Kutish, G.F., Tulman, E.R., French, R.A., Sur, J.H., Rock, D.L., Borca, M.V., 2005b. Mutation of E_1 glycoprotein of classical swine fever virus affects viral virulence in swine. Virology 343, 116–127.

Risatti, G.R., Holinka, L.G., Carrillo, C., Kutish, G.F., Lu, Z., Tulman, E.R., Sainz, I.F., Borca, M.V., 2006. Identification of a novel virulence determinant within the E_2 structural glycoprotein of classical swine fever virus. Virology 355, 94–101.

Risatti, G.R., Holinka, L.G., Fernandez Sainz, I., Carrillo, C., Kutish, G.F., Lu, Z., Zhu, J., Rock, D.L., Borca, M.V., 2007a. Mutations in the carboxyl terminal region of E_2 glycoprotein of classical swine fever virus are responsible for viral attenuation in swine. Virology 364, 371–382.

Risatti, G.R., Holinka, L.G., Fernandez Sainz, I., Carrillo, C., Lu, Z., Borca, M.V., 2007b. N-linked glycosylation status of classical swine fever virus strain Brescia E_2 glycoprotein influences virulence in swine. J. Virol. 81, 924–933.

Rumenapf, T., Unger, G., Strauss, J.H., Thiel, H.J., 1993. Processing of the envelope glycoproteins of pestiviruses. J. Virol. 67, 3288–3294.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

Schneider, R., Unger, G., Stark, R., Schneider-Scherzer, E., Thiel, H.J., 1993. Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science 261, 1169–1171.

Thiel, H.J., Stark, R., Weiland, E., Rumenapf, T., Meyers, G., 1991. Hog cholera virus: molecular composition of virions from a pestivirus. J. Virol. 65, 4705–4712.

Van Gennip, H.G., Vlot, A.C., Hulst, M.M., De Smit, A.J., Moormann, R.J., 2004. Determinants of virulence of classical swine fever virus strain Brescia. J. Virol. 78, 8812–8823.

Weiland, F., Weiland, E., Unger, G., Saalmuller, A., Thiel, H.J., 1999. Localization of pestiviral envelope proteins E(rns) and E_2 at the cell surface and on isolated particles. J. Gen. Virol. 80 (Pt 5), 1157–1165.

Zsak, L., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L., 1996. An African swine fever virus virulence-associated gene *NL-S* with similarity to the herpes simplex virus *ICP34.5* gene. J. Virol. 70, 8865–8871.